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(57) Abstract

The present invention provides a novel high efficiency method for the cell free engineering and synthesis of protein. A novel method of the present invention comprises the steps of: preparing a cell free extract; separating out a ribosome fraction from said extract; incubating said ribosome fraction in the presence of a transcription/translation medium; and measuring the amount of protein synthesized. The method of the present invention may be used as a coupled transcription/translation system, a translation only system or a cell-free continuous flow system. Also provided are methods for synthesis of proteins and their correct folding using chaperone proteins.

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CELL FREE SYSTEM FOR PROTEIN SYNTHESIS AND USE OF CHAPERONE PROTEINS THEREIN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the field of protein synthesis. More specifically, the present invention relates to a high efficiency system for cell-free translation of desired proteins or cell-free, coupled transcription/translation of exogenously added genes.

Description of the Related Art.

Cell-free translation of coding sequences cloned into plasmids circumvents many of the problems that may be encountered with gene expression in intact cells. These problems include product insolubility, toxicity, and difficulties in subsequent purification. The primary deficiency of cell-free protein synthesis has been the relatively small amount of product that is formed. This deficiency appeared to be largely overcome with the development by Spirin and his co-workers, *Science* 242:1162 (1988) of continuous-flow cell-free (CFCF) translation and coupled transcription/translation systems in which a relatively high level of protein synthesis could be maintained at a linear rate for an extended period of time, *i.e.*, 24 hours or longer. For example, a high rate of protein synthesis can be maintained for 24 hours in a coupled transcription/translation CFCF system derived from *E. coli*.

A cell-free extract from *Escherichia coli* (S30) suitable for translation was developed by G. Zubay (1973)

Ann. Rev. Genetics 7:267-287. The S30 extract is essentially free of DNA and devoid of mRNA due to preincubation under translation conditions but retains most of the soluble proteins and RNAs of intact cells. The use of the S30 system for coupled transcription/translation of linearized phage or plasmid DNA and linearized DNA fragments has been demonstrated (J. M. Pratt et al. (1981) Nucl. Ac. Res. 9:4459-4474; and J. M. Pratt et al. (1984) Transcription and Translation: A practical Approach (Hames, B. D., and Higgins, S. J., eds.), pages 179-209, IRL Press).

However, a number of laboratories that have attempted to carry out CFCF translation have had difficulties in establishing this technically complex system. The original system for coupled transcription/translation is based on the crude S30 fraction using the method of Zubay. In addition to the ribosomes, this fraction contains nearly all of the soluble protein that is present in intact *E. coli* cells. This includes many degradative proteases and nucleases. A specific consequence of this crude system is that the filter of the reaction chamber tends to clog during the CFCF run, apparently due, at least in part, to denatured protein that is formed from the soluble protein in the S30 fraction during the run. Secondly, the S30 fraction appears to contain one or more very active soluble exo-deoxyribonucleases that degrade linear DNA from its free end. Thirdly, the protein and nucleic acid factors required for the cell-free synthesis of enzymatically active proteins are poorly defined and characterized. Little is known of how these factors interact with each other and with the ribosomes during protein synthesis.

It is generally accepted that information for proper folding of a nascent protein is inherent in the

coding sequence of its gene . However, the questions of whether a nascent polypeptide folds on the ribosome as it is being formed vectorially from its N-terminus to C-terminus or whether folding into its native conformation takes place only after its synthesis has been completed, i.e. is folding of a newly formed polypeptide cotranslational or posttranslational, have not been answered. Some parts of the folding process may occur cotranslationally whereas others might occur during or after release of the newly formed polypeptides from the ribosomes and the process may be different for different proteins.

The initial folding of the nascent peptides may occur as they are extended on ribosomes. These folding events may take place in a protected area within a cavity or tunnel in the large ribosomal subunit. Chaperones, DnaJ, DnaK, GrpE, GroEL and GroES, may be involved in the folding of some proteins on ribosomes.

The prior art remains deficient in a high efficiency, coupled transcription/translation system for the cell-free engineering and synthesis of proteins. The prior art is also deficient in a high efficiency translation system for the cell-free synthesis of proteins.

SUMMARY OF THE INVENTION

The present invention fulfills a much-needed and longstanding need in the art of protein synthesis for a high efficiency, coupled transcription/translation system for the cell-free engineering and synthesis of proteins. The present invention also fulfills the longstanding need in this art for a high efficiency, cell-free translation system for the synthesis of proteins.

In one embodiment of the present invention

there is provided a method for the high efficiency, cell free synthesis of proteins, comprising the steps of: preparing a cell free extract; separating out a ribosome fraction from said extract; incubating said ribosome fraction in the presence of a transcription/translation medium; and measuring the amount of protein synthesized.

In another embodiment of the present invention, there is provided a method for the high efficiency, cell free synthesis of proteins, comprising the steps of: preparing a cell free extract; separating out a ribosome fraction from said extract; incubating said ribosome fraction in the presence of a transcription/translation medium containing chaperone proteins; and measuring the amount of protein synthesized.

In yet another embodiment of the present invention, there is provided a method for the high efficiency, cell-free synthesis of proteins, comprising the steps of: preparing a cell-free extract from *Escherischia coli;* separating out a ribosome fraction from said extract by centrifugation; incubating said ribosome fraction in the presence of a medium, said medium comprising 55 mM Tris-acetate (pH 7.8), 12 mM $Mg(OAc)_2$, 36 mM NH_4OAc, 72 mM KOAc, 2 mM $Ca(OAc)_2$, 0.5 mM EDTA, 2% polyethylene glycol-6000, 2 mM DTT, 1.2 mM ATP, 0.8 mM GTP, 0.8 mM UTP, 0.8 mM CTP, 0.4 mM cAMP, 27 mM phosphoenol pyruvate (monopotassium salt, pH 7.0), 0.35 μg pyruvate kinase, 1 μg folinic acid , 83 μM ^{14}C-leucine, 330 μM of each of the other 19 maino acids, 20 μg E. coli tRNA, 0.5 μg rifampicin, 0.3 mM glucose-6-phosphate, 1.2 A_{260} units of the *E. coli* ribosome fraction 0.5 μg plasmid DNA and 0.5 μg SP6 RNA polymerase; and measuring the amount of protein synthesized. Alternatively, the chaperone proteins DnaJ, DnaK, GrpE, GroEL and GroES may

be included in the medium to achieve the advantages set out below.

Other and further objects, features and advantages will be apparent from the following descriptions of the presently preferred embodiments in the invention which are given for the purpose of disclosure and when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope. The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

Figure 1 shows the enzymatic activity of the dihydrofolate reductase product. Closed circles and open triangles, respectively, are with the reaction mixtures that either contained or did not contain added pSP65 DHFR plasmid. Insert: A and B represent tracks from an autoradiogram derived from a 15% Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (performed according to Laemmli (1970) Nature 227:680-685) on which identical aliquots after *in vitro* protein synthesis were analyz d (A, without, and B with the plasmid). Numbers

1-5 indicate positions of marker proteins on the gel. These were: 1=phosphorylase b (M_r 94,000), 2=bovine serum albumin (M_r 65,000), 3= ovalbumin (M_r 43,000), 4=carbonic anhydrase (M_r 30,000), and 5=lysozyme (M_r 14,000).

Figure 2 shows the synthesis of dihydrofolate reductase in the continuous-flow cell-free system. Protein synthesis was followed over time by determining ^{14}C-leucine incorporation into protein. Fractions of 1.5 ml were collected per hour. Insert A: The Coomassie blue-stained gel (15% acrylamide) of individual fractions from the first 7 hours (100 μl from each). Insert B: Autoradiogram prepared from a gel similar to the one shown in Insert A with samples collected up to 22 hours.

Figure 3 shows the degradation of the linearized in contrast to the circular plasmid.

Figure 4 shows the rhodanese polypeptides in the supernatant and ribosome fractions. Rhodanese was synthesized *in vitro* in the presence of [^{14}C]leucine (160 Ci/mol) or [^{35}S]fMet-tRNA$_f$ (400 Ci/mol), then the ribosomes were collected by centrifugation. The supernatant and the resuspended ribosome fraction were analyzed by SDS-PAGE followed by autoradiography. Tracks 1 & 2 = 20 μl of the supernatant fractions; tracks 3 & 4 = 20 μl of the ribosome fractions (resuspended in 30 μl). Tracks 1 & 3 = from incubations with [^{14}C]leucine; tracks 2 & 4 = from incubations with [^{35}S]fMet-tRNA$_f$. The arrow on the left indicates migration of native rhodanese purified from bovine liver. The numbers on the right indicate positions of molecular weight markers; these are 1 = bovine serum albumin (M_r 64,000); 2 = ovalbumin (M_r 43,000); 3 = carbonic anhydrase (M_r 30,000); 4 = soybean trypsin inhibitor (M_r 22,000); 5 = lysozyme (M_r 14,400).

Figure 5 shows the release of full-length rhodanese from the ribosomes by chaperones. Resuspended ribosomes bearing rhodanese polypeptides were incubated in the absence or presence of the indicated chaperones (amounts given in TABLE VII), then centrifuged. Aliquots of 20 μl of the supernatant (first lane of each set) and 20 μl of the ribosome fraction that had been resuspended in 30 μl (each second lane) were analyzed by SDS-PAGE and autoradiography. The fraction of the full-length rhodanese released into the supernatant relative to the total amount of full-length rhodanese originally bound to the ribosomes is given as percentage beneath the tracks. The amount of full-length rhodanese was determined by cutting out and solubilizing the band from the gel, then counting the radioactivity in the presence of Ecolite (ICN).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the term "S30" refers to a cell-free extract from *E. coli* prepared according to Zubay (1973). The preparation of a cell free extract from E. coli is a well known procedure in the prior art and can readily be accomplished by a person having ordinary skill in this area of research.

As used herein, the term "cell free translation" refers to *in vitro* synthesis of a protein.

As used herein, the term "continuous flow, cell free or CFCF system" refers to synthesis of a protein in a reaction chamber from which product is pumped out and feeding solution is pumped in continuously similar to the CFCF system described by Spirin et al. (1988). Briefly, a continuous flow of feeding buffer, including amino acids and nucleotide triphosphat s is used through a reaction mixture. There is continuous removal of the

polypeptide product. Both prokaryotic and eukaryotic systems were tested.

As used herein, the term "coupled transcription/translation" refers to synthesis of a mRNA from a plasmid i.e., transcription and translation in the same reaction mixture containing this mRNA.

As used herein, the term "ribosome fraction" refers to a fraction derived from the crude cell extract which contains the ribosomes and certain proteins, tRNA and other cellular components.

As used herein, the term "static assay or static system" refers to an assay carried out in a test tube and is not a continuous flow system.

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The present invention provides a high efficiency method for the cell free synthesis of proteins. This method comprises the steps of: (1) preparing a cell free extract; (2) separating out a ribosome fraction from the extract; (3) incubating the ribosome fraction in the presence of a medium; (4) and measuring the amount of protein synthesized.

The cell free extract prepared for use in the method of the present invention may be prepared from many prokaryotic or eukaryotic organisms. A representative example of suitable prokaryotic organisms is *Escherichia coli*. Representative examples of suitable eukaryotic organisms include wheat germ and rabbit reticulocytes.

In the method of the present invention, the ribosome fraction may be separated from the cell free extract by any of the well known methods in the art.

Preferably, the ribosome fraction is separated by centrifugation or gel filtration chromatography.

In the method of the present invention, the ribosome fraction is incubated in the presence of an appropriate medium. In one embodiment of the present invention, the ribosome fraction is incubated in the presence of a transcription/translation medium. Generally, the transcription/translation medium contains buffer, salts (with monovalent and divalent cations), amino acids, reducing agent, nucleoside triphosphates, cell extract (S30) or ribosomes, a plasmid, RNA polymerase, and a energy regenerating system. Most preferably, the transcription/translation medium contains Hepes or Tris buffer (pH 7.5-7.8), optimal $Mg(OAc)_2$ concentration, optimal NH_4^+ and/or K^+ salt concentration, 2 mM DTT, 1.2 mM ATP, 0.8 mM each of GTP, UTP, and CTP, 0.5 mM cAMP, energy regenerating system (either phosphoenol pyruvate and pyruvate kinase or creatine phosphate and creatine phosphate kinase), 1 μg folinic acid (only for *E. coli* system), 25-83 μM ^{14}C-leucine, 25-330 μM of each of the other 19 amino acids, 20-30 μg tRNA (prokaryotic tRNA for *E. coli*; eukaryotic tRNA for eukaryotic systems), 0.5 μg rifampicin, 1.2 A_{260} units of the ribosome fraction, plasmid DNA, and RNA polymerase. The optimal concentration of $Mg(OAc)_2$, NH_4^+ and K^+ will vary depending on whether prokaryotic or eukaryotic organisms are used. For example, the K^+ concentration used with bacterial system was 72 mM but was 112 mM in the wheat germ system. Similarly, the Mg^{2+} concentration in the bacterial system was 14 mM while it was 4 mM in the wheat germ system. A person having ordinary skill in this art would readily recognize that the concentration of various salts may vary slightly from laboratory to laboratory.

In another embodiment of the present invention, the ribosome fraction is incubated in the presence of a translation medium. Generally, the translation medium contains buffer, salts, source of energy e.g., ATP and GTP, an energy regenerating system e.g., phosphoenol pyruvate, pyruvate kinase, amino acids, tRNAs, and isolated mRNA.

In another embodiment of the present invention, there is provided a high efficiency method for the cell free synthesis of proteins, comprising the steps of: preparing a cell free extract; separating out a ribosome fraction from said extract; incubating said ribosome fraction in the presence of a transcription/translation medium containing chaperone proteins; and measuring the amount of protein synthesized. Generally, the chaperones preferably included in the medium are DnaJ, DnaK, GrpE, GroEL and GroES.

In the method of the present invention, the ribosome fraction of the cell free extract is incubated in the medium at about 37°C for about 20 minutes to about 60 minutes. Preferably, the incubation time is about 30 minutes at 37°C.

The protein synthesized by the methods of the present invention may be determined by any of the well known techniques. Preferably, protein is measured by trichloroacetic acid precipitation followed by quantitation of the amount of amino acid incorporated into the protein or analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. Also, biological activity (like

enzymatic activity) is determined where applicable and reaction with specific antibodies (if these are available) may be carried out.

In some cases, it may be necessary or desirable to determine protein levels by measuring the biological activity of the synthesized protein. In other words, the native conformation of the protein may be determined by measuring the biological activity of the protein.0 Representative examples of proteins that can be measured by examining the protein's biological activity includes rhodanese, chloramphenicol acetyl transferase and dihydrofolate reductase.

Ribosomes separated from the S30 *E. coli* extract retain all the components necessary for coupled transcription/translation from a plasmid which contains the coding sequence under an appropriate promoter. A considerably larger amount of synthesis was obtained with the nonlinearized plasmids and the linearized forms most often used in the past were rapidly degraded in the system. The utility of the system was shown for both prokaryotic and eukaryotic proteins. The present invention has important advantages over any prior art system.

Plasmid preparations were made by standard procedures (J. Sambrook et al. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press) except that the CsCl centrifugation step was replaced by Q cartridge (Bio-Rad) chromatography. SP6 and T7 RNA polymerases are commercially available. The plasmids used are described in the following references: (1) Coding sequence: β-lactamase/DHFR; plasmid pDF34-DHFR (Baranov et al. (1989) *Gene* 84:463-466); (2) Coding sequence: DHFR; plasmid SP65-DHFR; (Murzina and Gudkov (1990) *Prot. Engineering* 3:709-712); (3) Coding sequence: CAT;

plasmid SP65-CAT; provided by A. Spirin and co-workers; (4) Coding sequence: PP1-C; plasmid pGEM-3Z-PP1-C; (Bai et al. (1988) *FASEB J.* 2:3010-3016); (5) Coding sequence: STNV; plasmid pTZ19R-STNV, (Browning et al. (1988) *J. Biol. Chem.* 263:8380-8383); (6) Coding sequence: rhodanese; plasmid pET11d-rho, (Miller et al. (1991) *J. Biol. Chem.* 266:4686-4691); (7) Coding sequence: hTFIID-180C; plasmid pAR-180C, (Peterson et al. (1991) *Science* 248:1625-1630); (8) Coding sequence: β-globin; plasmid pUC18, (Fletcher et al. (1990) *J. Biol. Chem.* 265:19582-19587).

EXAMPLE 1

Isolation of the ribosome fraction

The ribosome fraction used for coupled transcription/translation was isolated from the S30 fraction prepared according to G. Zubay from *E. coli* K12 (A19). Cells were grown at 37°C in LB (Sigma) broth to which 20% glucose (10 ml per 2 liter medium) was added. Cells were harvested in mid-log phase at 37°C and lysed in a pressure cell. The cells were then centrifuged for 30 minutes at 30,000 x g and the S30 extract was prepared. A protease inhibitor, phenylmethylsulfonyl fluoride, was added to the lysis buffer to give a final concentration of 0.5 mM. Aliquots of the S30 (9 ml each) were centrifuged at 47,000 rpm for 4 hours in a Beckman Ti 50 rotor. The sedimented ribosomes were resuspended in 1.0 ml of 20 mM Tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$, and 1 mM DTT resulting in a suspension of about 1200 A_{260} units/ml. This ribosome fraction was stored at -70°C in small aliquots.

EXAMPLE 2

Synthetic System

The system used to carry out coupled transcription/translation contained in a total volume of

30 μl: 50 mM Tris-acetate (pH 7.8), 14 mM $Mg(OAc)_2$, 36 mM NH_4OAc, 72 mM KOAc, 2 mM $Ca(OAc)_2$, 0.5 mM EDTA, 2% polyethylene glycol-6000, 2 mM DTT, 1.2 mM ATP, 0.8 mM each of GTP, UTP, and CTP, 0.5 mM cAMP, 27 mM phosphoenol pyruvate, 0.35 μg pyruvate kinase, 1 μg folinic acid, 83 μM ^{14}C-leucine, 330 μM of each of the other 19 amino acids, 20 μg *E. coli* tRNA (Boehringer), 0.5 μg rifampicin, 0.3 mM glucose-6-phosphate, 1.2 A_{260} units of the *E. coli* ribosome fraction, 0.5 μg plasmid DNA, and 0.5 μg SP6 RNA polymerase. ^{14}C-leucine was diluted to 40 Ci/mole for the static test tube assay and to 10 Ci/mole for the continuous-flow cell-free system. Incubation was for 30 minutes at 37°C. For the continuous-flow cell-free system, the reaction mixture was enlarged to 1.0 ml. Synthesis was carried out in a reaction chamber with the YM100 (Amicon) membrane on its upper side through which product was pumped bottom to top at 1.5 ml/hr. The eluate was replaced by feeding solution which contained all low molecular weight components of the reaction mixture and tRNA at 3 $\mu g/ml$. Incubation was at 37°C for the times indicated.

EXAMPLE 3

Analysis of the protein product

The product formed was analyzed in one of the following ways. The amount of ^{14}C-labeled product in the 30-μl test tube assay or from a 100-μl aliquot of the fractions collected from the continuous-flow system was determined by trichloroacetic acid precipitation following the procedure described previously (W. Kudlicki et al. (1987) J. Biol. Chem. 262:9695-9701). Radioactivity was quantitated by liquid scintillation counting. Alternatively, an aliquot of the reaction mixture or the eluate was analyzed by polyacrylamide gel electrophoresis in SDS followed by autoradiography (W.

Kudlicki et al. (1987) J. Biol. Chem. 262:9695-9701). The enzymatic activity of dihydrofolate reductase (DHFR) was determined according to Baccanari et al. (1981) Biochemistry 20:1710-1716 by oxidation of NADPH as indicated by decreased absorbance at 340 nm. One unit of DHFR activity is defined as the amount of enzyme required to reduce 1 μmol dihydrofolate/min based on a molar extinction coefficient of 12.3×10^3 for NADPH (B. L. Hillcoat et al (1967) Anal. Biochem. 21:178-189).

Enzymatic activity of rhodanese was determined in a calorimetric assay according to Sorbo (1953), Acta Chem. Scand. 7:1137-1145. This assay measures the conversion of CN^- to SCN^- by the enzyme using $S_2O_3^{2-}$ as substrate. SCN^- formed was detected and quantitated by measuring absorbance at 460 nm of a complex between this product and ferric ions. One unit is defined as the amount of enzyme generating 1 μmol product/minute at 37° C in this assay system. Chloramphenicol acetyl transferase (CAT) activity was measured by the transfer of [^{3}H]acetyl from acetyl-CoA to chloramphenicol attached to agarose beads. Radioactivity associated with the beads after the enzyme reaction was quantitated. One unit was defined as the transfer of 1 pmol ^{3}H-acetyl to chloamphenicol.

EXAMPLE 4

The ribosome fraction used in the experiments was derived from an *E. coli* S30 fraction. Either the S30, or the ribosome fraction, was incubated with plasmids which contained a coding sequence under the SP6 or T7 promoter unless otherwise indicated. SP6 or T7 RNA polymerase was added to carry out transcription from the respective promoter. The 30 μl transcription/translation reaction mixtures also were supplemented with tRNA and the low molecular weight components necessary to carry

out RNA and protein synthesis. When the static assay was used, incubations were carried out for 30 minutes at 37°C.

Protein synthesis was carried out as follows: Reaction mixtures contained either 10 μl S30 or 1 μl ribosome fraction. In plasmids 2, 3 and 4, from which the genes listed in lines 2-4 of Table 1 were transcribed, the respective coding sequence was under the SP6 promoter. In these cases, SP6 RNA polymerase and rifampicin were added. In plasmid 5, the DHFR fol A gene was inserted into a plasmid (pDF34 - (V. I. Baranov et al. (1989) Gene 84:463-466)). Transcription of this gene as well as of β-lactamase is under the *E. coli* promoter, i.e., no RNA polymerase or rifampicin were added. The plasmids whose genes are listed in lines 6-8 contain the coding sequence under the T7 promoter. In these cases, T7 RNA polymerase and rifampicin were added. The amount of protein synthesized was analyzed by incorporation of ^{14}C-leucine.

An aliquot of the reaction mixture containing 50 ng of DHFR protein synthesized in the static system (determined from the amount of ^{14}C-leucine incorporated into protein) was used to measure enzymatic activity. An equal aliquot was taken from a parallel incubation mixture from which the plasmid was omitted. To determine enzymatic activity, the assay mixture contained in a total volume of 1.0 ml, 100 mM imidazole-HCl (pH 7.6), 10 mM 2-mercaptoethanol, 75 μM dihydrofolate, and 80 μM NADPH. The reaction carried out at 30°C was followed in the cuvette by the decline in absorbance at 340 nm.

An aliquot of the reaction mixture in which rhodanese was synthesized was used to determine its activity. About 150 ng rhodanese (determined from the amount of ^{14}C-leucine incorporated into protein) was added

to a 1:1:1 mixture (final volume 0.5 ml) of 0.15 M thiosulfate, 0.15 M KCN and 0.12 M KH_2PO_4 (pH 5). The reaction was carried out at 37° C; it was stopped by the addition of 0.25 ml 18% formaldehyde. The product formed was detected after admixing 0.75 ml 0.15 M $Fe(NO_3)_3$ in 33% HNO_3. The absorbance at 460 nm of the final reaction mixture was used to determine its activity. About 30 ng CAT (determined from the amount of ^{14}C-leucine incorporated) was added to a mixture containing [^3H]acetyl-CoA (3000 Ci/mol) and chloramphenicol bound to agarose beads. The assay was carried out at 25°C, the beads were washed and their radioactivity determined. Alternatively, the method of Sleigh et al., *Analyt. Biochem.* 156:251-256 (1986) was used which measures ^{14}C acetyl-CoA transfer to chloramphenicol after one hour incubation at 37° C under the conditions described by Sleigh et al.

EXAMPLE 5

Incorporation of ^{14}C-leucine into protein was nearly linear over the time of incubation but stops quite abruptly at 30 minutes so that maximal or nearly maximal synthesis is obtained by this time. The basis for this sudden rate decrease is not known. The amount of protein synthesized during this period from different plasmids in either the S30 or in the ribosome fraction is shown in TABLE I. With the fractionated system, background synthesis is lower with nearly all plasmids tested.

Data for plasmids with the following coding sequences inserted are given in TABLE I: *E. coli* dihydrofolate reductase (DHFR), *E. coli* chloramphenicol acetyl transferase (CAT) and rabbit skeletal muscle phosphatase (the catalytic subunit of type 1 phosphoprotein phosphatase, PP1-C). The coding sequences for these three proteins had been cloned into different

plasmids but all are under the SP6 promoter. With these plasmids, SP6 RNA polymerase was added for transcription. In these cases, rifampicin was added to the reaction mixture to inhibit the endogenous *E. coli* RNA polymerase. Also included in TABLE I are results obtained with a plasmid into which the DHFR gene was inserted under the *E. coli* promoter. In this case, transcription was performed without added RNA polymerase. Two products were synthesized, DHFR and a polypeptide of 30,000 daltons, which appears to be β-lactamase. The latter product was also obtained when the other SP65 plasmids were transcribed and translated in the absence of rifampicin. The plasmids listed in TABLE I that have the inserted coding sequence under the T7 promoter are: bovine rhodanese, a truncated form of the human TATA box binding protein (TFIID-180C) and a plant viral RNA from Satellite Tobacco Necrosis Virus (STNV). With these plasmids, T7 RNA polymerase and rifampicin were added for transcription.

TABLE I

Comparison of *in vitro* protein synthesis in the *E. coli* S30 and the ribosome fraction derived from it.

	Genes added	Protein synthesized in 30-μl assay (^{14}C-leucine incorporated, pmol) S30	ribosome fraction
1.	none	9.7	4.2
2.	DHFR	200.7	240.3
3.	CAT	285.4	305.6
4.	PP1-C	156.0	159.4
5.	β-lact/DHFR	270.2	320.6
6.	rhodanese	473.1	613.0
7.	TFIID-180C	534.0	n/d
8.	STNV	386.3	440.1

EXAMPLE 6

Most importantly, the plasmids used for the experiments in TABLE I were not linearized for transcription by either added SP6 or T7 polymerase or endogenous *E. coli* RNA polymerase. A lower, in some cases very much lower level of translation was obtained provided the plasmid had been cut 3′ of the coding sequence by an appropriate restriction enzyme (TABLE II). Degradation of the linearized plasmid (in contrast to the uncut plasmid) was observed during the incubation time as analyzed by agarose gel electrophoresis (as is shown in Figure 1).

Protein was synthesized in the fractionated system. PP1-C was linearized with Not I (restriction site was located about 240 base pairs downstream from the stop codon as described by Bai et al (1988). Both DHFR (short) and DHFR (long) were linearized with Hind III.

The influence of the 3' untranslated region of the transcribed sequence was also seen. The DHFR "short" plasmid contains only 165 base pairs between the stop codon and the restriction site (Hind III) used for linearization. The DHFR "long" plasmid has an additional 350 base pairs (515 total) in this 3' untranslated region. The lower level of translation may be due to 3' exonucleolytic activity that degrades the plasmid and is present even in the ribosome fraction.

TABLE II

Advantage of circular plasmid DNA for *in vitro* protein synthesis

	Protein synthesized (ug/ml reaction mixture)		
Plasmid added	DHFR (short)	DHFR (long)	PP1-C
linearized	2.36	7.53	0.06
non-linearized	13.40	9.58	5.20

EXAMPLE 7

The methods of the present invention may also be carried out using a cell-free extract obtained from eukaryotic organisms. Protein synthesized from either an S30 or the ribosome fraction of an S30 extract from wheat germ (TABLE III) or rabbit reticulocyte lysate (TABLE IV).

The wheat germ system used to carry out coupled transcription/translation contained in a total volume at 50 μl having 25 mM Hepes-KOH (pH 7.6), 2.4 mM DTT, 0.1 mM spermine, 1.2 mM ATP, 1.0 mM GTP, 0.8 mM CTP, 0.8 mM UTP, 1.0 mM GMP, 6 mM creatine phosphate, 28 U creatine phosphokinase, 215 μM each amino acid (excluding leucine), 15 μg wheat germ tRNA's, 112 mM KOAc, 4 mM $Mg(OAc)_2$, 50 U human placenta ribonuclease inhibitor, 0.1 μg each protease inhibitor (aprotinin, leupetin and

pepstatin), 2 - 2.2 μg plasmid, 27 U(~0.5 μg) T7 RNA polymerase, 25 μM[^{14}C]leucine diluted to 40 Ci/mol, 12 μl of wheat germ S30 or 1.5 A_{260} units of ribosomes.

TABLE III

Comparison of *in vitro* protein synthesis in the wheat germ S30 and ribosome fraction derived from it

Genes added		Protein synthesized ug/ml reaction mixture	
		S30	ribosome fraction
1.	STNV	8.5	7.3
2.	β-globin	4.0	3.5

Rabbit reticulocyte lysate treated with micrococcal nuclease was prepared as described by Pelham and Jackson (1976) Eur. J. Biochem. 67:247-256. After a 4 hour ultracentrifugation, ribosomes were resuspended in 20 mM Tris-HCl (pH 7.5), 1.2 mM $MgCl_2$, 100 mM KCl, and 1 mM DTE. The reaction mixtures of 25 μl contained about 50% (v/v) nuclease-treated lysate or 1.5 A_{260}-units of ribosomes isolated from the lysate and additions to give the following concentrations: 10 mM Tris-HCl (pH 7.5), 1.0 mM ATP, 0.8 mM GTP, CTP and UTP, 5 mM creatine phosphate, 75 μg/ml creatine phosphokinase, 0.05 mM each unlabeled amino acid (excluding leucine), 3.3 mM $MgCl_2$, 80 mM KCl, 500 units/ml T7 RNA polymerase, 15 μg/ml plasmid DNA, [^{14}C]leucine (10-40 Ci/mol), 50 U human placenta ribonuclease inhibitor. Incubation was at 35°C for 30-60 minutes.

TABLE IV

Comparison of *in vitro* protein synthesis in the rabbit reticulocyte lysate and ribosome fraction derived from it

Genes added		Protein synthesized ug/ml reaction mixture	
		S30	ribosome fraction
1.	β-globin	8.7	6.2
2.	STNV	2.78	2.3

TABLE IV

The results from TABLES III and IV demonstrate that the amount of protein synthesized from the ribosome fraction derived from rabbit reticulocyte lysate and wheat germ is somewhat quantitatively less than the amount of protein synthesized from the S30 extract. However, using the ribosome fraction in a eukaryotic system in the methods of the present invention is still desirable for several reasons. First of all, use of the ribosome fraction in the methods of the present invention provides a "cleaner" system for the in vitro synthesis of proteins. That is, the system is not contaminated with proteins contained the S30 extract. Thus, the use of the ribosome fraction significantly improves the methods of the present invention in the CFCF system. Secondly, a person having ordinary skill in this art would readily recognize that the elements of the use of the ribosome fraction from eukaryotic organisms may be varied to increase the amount of protein synthesized.

EXAMPLE 8

The plasmid containing the "short" DHFR was used in the uncut form for the following experiments. Coupled transcription/translation was carried out in the

presence of ^{14}C-leucine in the static system. An aliquot of the reaction mixture was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Another aliquot was used to determine enzymatic activity of the translation product. The results are shown in Figure 2. The insert indicates a full-length product as the only radioactive band seen in the autoradiogram. Enzymatic activity was determined by the decrease in absorbance at 340 nm due to oxidation of NADPH.

Enzyme activity of the translation product over time is shown in Figure 2. From other experiments, the specific activity of the *in vitro*-synthesized DHFR was calculated to be about 55 units/mg protein which compares favorably with isolated homogeneous DHFR. Background DHFR activity was low in the fractionated transcription/translation system, whereas it was at least ten fold higher in the original S30 extract. At this level, it was difficult to measure the enzymatic activity of the *in vitro* synthesized DHFR. These experiments were carried out in a 30-μl static transcription/translation system.

TABLE V presents data concerning the enzymatic activity of in vitro synthesized rhodanese and CAT. In both cases, background values are provided to indicate that the protein-synthesizing system does not contain activities that would interfere with the respective assay.

TABLE V

Enzymatic activity of *in vitro* synthesized rhodanese and CAT

	protein synthesis (pmol/30 ul assay)	specific enzymatic activity (units/mg)
rhodanese		
without plasmid	0.3	4.48
with plasmid	8.5	327
	protein synthesis (pmol/30 ul assay)	enzymatic activity (pmol 3H-acetyl transferred/min/pmol)
CAT		
without plasmid	0.6	430
with plasmid	8.4	3330

EXAMPLE 9

Synthesis of DHFR from the non-linearized SP6 plasmid in the CFCF system is shown in Figure 3. The reaction chamber of the cell used was such that product and expended reactants were continuously removed by pumping, usually at a rate of 1.5 ml/hr, and replaced by feeding solution containing nucleoside triphosphates, amino acids, tRNA, and the appropriate salt concentrations. The reaction mixture is identical to that in the 30-μl static assay except that a volume of 1 ml is used. Figure 3 indicates that protein synthesis was continuous over the period tested (22 hours) as indicated by ^{14}C-leucine incorporation into total protein. The analysis by SDS polyacrylamide gel electrophoresis and autoradiography of aliquots tak n during the time

period is shown in inserts A and B of Figure 2. Coomassie blue staining of the gel shows that a high proportion of the soluble *E. coli* proteins from the S30 that remain in the ribosome fraction are removed from the reaction chamber during the first 3 hours of incubation. Nearly pure product is obtained during the subsequent incubation period as seen on the autoradiogram.

The present invention demonstrates that the ribosomal synthesis of proteins can be maintained at a linear rate during many hours of incubation in the CFCF system. The modifications of the classical *E. coli* S30 system of Zubay in the present invention have two major advantages for expression of genes by coupled transcription/translation in either the static or CFCF system.

Fractionation of the S30 removes most of the soluble components of the *E. coli* extract that may complicate subsequent purification or assays of the product. Also, they may directly inhibit transcription or translation. The fractionated system is much less viscous than the S30 extract. Turbidity due to denatured protein in the reaction mixture is greatly reduced with the fractionated system even after many hours of incubation in the CFCF reaction chamber. In the CFCF system, clogging of the membrane due to denatured protein and degraded nucleic acids is greatly reduced.

Secondly, much greater stability of the transcription system is obtained with the circular plasmid. The use of a restriction enzyme to linearize the plasmid is obviated. The coupled system derived from *E. coli* works well for the eukaryotic proteins tested. Capping of the mRNA for eukaryotic proteins appears to be unnecessary with the *E. coli* system.

Furthermore, eukaryotic cell-free systems can be prepared in a similar way by isolating the ribosome fraction from wheat germ extract or reticulocyte lysate. That is, the ribosome fraction derived from these eukaryotic cells (TABLES III and IV) contains all of the components required for coupled transcription/protein synthesis. In contrast to the system derived from *E. coli*, with the eukaryotic cell extract or with the ribosome fractions derived from them, efficient translation can be carried out with mRNAs added directly to the reaction mixture, i.e., without coupled transcription (TABLE VI).

Using the wheat germ translation system in TABLE VI, the reaction mixture in a total volume of 50 ul contains 25 mM Hepes-KOH (pH 7.6); 2.4 mM DTT; 0.1 mM spermine; 1.2 mM ATP; 1.0 mM GTP; 6 mM creatine phosphate; 28 U creatine phosphokinase; 25 uM each amino acid (excluding leucine); 15 μg wheat germ tRNA; 112 mM KCl; 2 mM $Mg(OAc)_2$; 2-2.5 μg mRNA; and 12 μl wheat germ S30 or 1.5-1.7 A_{260} units of ribosomes. Incubation was for 30 minutes at 27°C.

Using the reticulocyte translation system in TABLE VI, the reaction mixture (25 μl) contains 10 mM Tris-HCl (pH 7.5); 1.2 mM $MgCL_2$; 90 mM KCl; 5 mM DTT; 0.5 mM ATP; 0.2 mM GTP; 50 μM each amino acid (except leucine); 3 mM creatine phosphate; 0.2 mg/ml creatine phosphokinase; 8.5 pmol mRNA; 12.5 μl reticulocyte lysate or 1.7 A_{260} units of ribosomes. Incubation was for 30 minutes at 35°C.

TABLE VI

Protein synthesis with lysates or "ribosome fractions" derived from wheat germ and rabbit reticulocytes to which isolated mRNA was added.

Material analyzed	Protein synthesized (ug/ml reaction mixture)
rabbit reticulocyte	
(Beta-globin synthesized)	
lysate	9.4
ribosome fraction	8.83
wheat germ	
dihydrofolate reductase	
lysate	2.5
ribosome fraction	2.2

The factors that are required for the initiation, elongation, and termination of peptides as well as the aminoacyl-tRNA synthetases are generally associated with ribosomes. The present invention indicates that the *E. coli* RNA polymerase also sediments with the *E. coli* ribosomes obtained from the DNA-free S30 fraction.

Another advantage of the efficient, *in vitro*, protein-synthesizing system of the present invention is that tRNAs with modified amino acids (for example, amino acids with covalently attached fluorophores) can be incorporated into nascent peptide chains. Also, synthetic tRNA can be added whose anticodon/amino acid relationship has been changed to allow *in vitro* protein engineering.

EXAMPLE 10

Propagation of the plasmid, isolation of SP6 RNA polymerase, preparation of the *E. coli* cell-free extract (S30) and isolation of the ribosome fraction from the S30 were carried out as described above. The rhodanese coding sequence was removed from the pET11d vector and ligated into the XbaI-BamHI sites of pSP65. This positions the rhodanese coding sequence under the promoter for SP6 RNA polymerase. Under the conditions used, translation was limited to rhodanese polypeptides. No β-lactamase can be synthesized from this plasmid when it was used in non-linearized form for coupled transcription/translation.

For analytical purposes, the synthesis of rhodanese was carried out in 30-μl reaction mixtures containing 5 mM $Na_2S_2O_3$. For large scale preparations, reaction mixtures were enlarged to 0.9 ml. These reaction mixtures contained about 60 A_{260} of crude *E. coli* ribosomes and about 10-15 μg non-linearized plasmid. [^{14}C]Leucine was used at 40 Ci/mol or 160 Ci/mol. Incubation was for 40 minutes at 37°C. In some experiments (Figure 4), the radioactive precursor was precharged formylated [^{35}S]Met-tRNA$_f$ (400 Ci/mol). In this case, folinic acid was omitted. After incubation, the reaction mixture was loaded over 0.6 ml buffered sucrose solution containing the same salt concentrations as used in coupled transcription/ translation, then was centrifuged in a Ti50 rotor (Beckman) for 45 minutes at 45,000 rpm. After centrifugation, the supernatant (0.9 ml) was removed, the sucrose layer discarded and the ribosomal pellet rinsed with the same solution in which it was subsequently resuspended (60 μl of 20 mM Tris-HCl, pH 7.5, 10 mM Mg $(OAc)_2$, 30 mM NH_4OAc, 1 mM DTT, 5 mM $Na_2S_2O_3$ comprised solution A). Aliquots of 25 μl from the

supernatant and 2 μl from the resuspended pellet, respectively, were removed to determine incorporation of [^{14}C]leucine into protein and for analysis by SDS-PAGE and autoradiography. Also, 15 μl from the supernatant and 1.5 μl from the ribosome fraction were withdrawn for determination of enzymatic activity.

EXAMPLE 11

The resuspended ribosome fraction was tested for activation and/or release of bound rhodanese in the following way. Reaction mixtures of 30 μl contained 55 mM Tris-acetate (pH 7.8), 2 mM DTT, 1.2 mM ATP, 0.8 mM GTP, 36 mM NH_4OAc, 72 mM KOAc, 12 mM $Mg(OAc)_2$, 2 mM $Ca(OAc)_2$, 5 mM $Na_2S_2O_3$, 1.9% polyethylene glycol (M_r 6,000), 27 mM phospho(enol) pyruvate (monopotassium salt), 0.33 mM glucose-6-phosphate, 0.5 mM EDTA, 0.3 μg pyruvate kinase, and 3 μl resuspended ribosomes. Chaperones or antibiotics were added as follows: GroEL - 2.1 μg, GroES - 0.8 μg, DnaK - 1.5 μg, DnaJ - 0.5 μg, GrpE - 1 μg. The specific enzymatic activity of native rhodanese isolated from bovine liver was 684 units/mg protein. Incubation was for 30 minutes at 37°C, then the sample was centrifuges in an airfuge (Beckman) for 30 minutes at 104,000 rpm. After centrifugation, the supernatant was carefully removed, the ribosomal pellet rinsed then resuspended in 30 μl of solution A. Aliquots of the supernatant and the ribosome fraction, respectively, were analyzed for the amount of rhodanese by determining [^{14}C]leucine incorporation into protein, for size of rhodanese by SDS-PAGE and autoradiography and for enzymatic activity.

SDS-PAGE was carried out in 12% gels. The dried gel was exposed to Hyperfilm (Amersham) for about 48 hours. Rhodanese enzymatic activity was determined as before (Tsalkova et al., 1993) Basically, quantitation

was by absorbance at 460 nm (= 4200) generated from a complex formed between the product, thiocyanate, and ferric ions. One unit of enzymatic activity is defined as 1 μmol product formed per minute at 25°C. Incubation was for 10 minutes at 25°C. A blank containing the reaction mixtures for activation and release or for coupled transcription/translation from which the plasmid had been omitted gave absorbance at 460 nm of 0.03-0.04.

The phenylalanine analog of puromycin has been prepared previously by reacting N-carbobenzoxyphenylalanine with puromycin aminonucleoside using the mixed anhydride method of activation. The fluorenylmethyl-oxycarbonyl (FMOC) moiety was used rather than the carbobenzoxy moiety to protect the amino group of phenylalanine and activated the carboxyl group by converting it into the succinimidyl ester using the dicyclohexylcarbodiimide method. (^{3}H)-L-Phenylalanine was converted into the FMOC derivative as previously described (Carpino and Han, 1972, *J.Org. Chem.* 37:3404-3409). The final product was estimated to be about 95% pure by thin layer chromatography on silica gel in chloroform : methanol (9:1). The R_f of the main product in this solvent was about 0.55, almost identical to that of puromycin and about twice that of puromycin aminonucleoside. The product had an absorbance maximum at 274 nm, identical to that found for puromycin. Using a molar extinction coefficient of 20,000 reported for puromycin, the overall yield of product was about 50%, based on phenylalanine.

EXAMPLE 12

Ribosome-bound rhodanese is enzymatically inactive.

Rhodanese was synthesized on *E. coli* ribosomes in a cell-free system by coupled transcription/translation as described above. The

results are given in TABLE VII. After a 30 minute incubation at 37°C, aliquots of the reaction mixture were withdrawn to quantitate the amount of protein that was synthesized and to determine its enzymatic activity. The latter parameter is expressed as enzyme units, μmol of thiocyanate formed per minute. Under the conditions used, only one coding sequence was transcribed from the plasmid (pSP65), namely the mRNA for rhodanese. Incorporation of [^{14}C]leucine into peptides in reaction mixtures minus plasmid was less than 5% of the incorporation in the presence of the plasmid and has been subtracted as a blank. Thus, with knowledge of the amino acid sequence of rhodanese (24 leucine residues per chain), the molecular weight (33,000) and of the percentage of full-length product (nearly 100% for the supernatant fraction, Figure 4), the molar amounts of full-length protein that was synthesized was calculated from the [^{14}C]leucine that was incorporated. This value was used to calculate the specific enzymatic activity, enzyme units/mg of rhodanese.

The reaction mixture was centrifuged after incubation to separate the ribosomes from the soluble fraction. About 50% of the [^{14}C]leucine that was incorporated into polypeptides was recovered in the ribosome fraction. All of the enzymatic activity was found in the supernatant fraction. The specific enzymatic activity for the supernatant fraction, 564 units per mg of rhodanese (TABLE VII A, No chaperones added), was about 82 per cent of the value (specific activity of 684 units/mg) determined under identical conditions for the native enzyme isolated from bovine liver by others previously. TABLE VII A and Figure 4 illustrate that about half of the total newly formed polypeptides remains associated with ribosomes after 30

minutes of incubation. This time was near the end of the period of linear incorporation of leucine into polypeptides under the conditions used. This result raises the question of why there is an accumulation of apparently full-length but inactive rhodanese polypeptides on the ribosomes. Also, about 15% of the full-length peptides that were released into the soluble fraction are enzymatically inactive. The effect of the addition of purified chaperones to the system was tested with the results presented in TABLE VII B. A higher proportion of newly formed material was released into the soluble fraction (76% vs. 55% in the absence of added chaperones) and the specific activity of the released material (681 units/mg) was experimentally equivalent to that of rhodanese isolated from liver. However, the total synthesis decreased from 98 pmol of leucine incorporated to 57 pmol.

TABLE VII

Separation of active rhodanese from ribosome-bound inactive form after its synthesis

Analyzed fraction	[^{14}C leucine incorp. (pmol)	enzymatic activity (units x 10^{-3})	specific enzymatic activity (units/mg rhodanese)
A. No chaperones added:			
total reaction mixture	98	37.2	---1
supernatant	45	34.9	564
ribosomes	42	0.0	0
B. GroEL/ES.DnaK/J.GrpE added:			
total reaction mixture	57	38.1	---1
supernatant	38	35.6	681
ribosomes	12	1.1	66.7

[1] specific enzymatic activity was not calculated for this fraction since the product bound to ribosomes includes incomplete, short peptides as well as full-length chains that are enzymatically inactive (see Figure 4).

The newly formed polypeptides that were recovered in both the supernatant and with the

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[1]1 Specific enzymatic activity was not calculated for this fraction since the product bound to ribosomes includes incomplete, short peptides as well as full-length chains that are enzymatically inactive (see Figure 1).

resuspended ribosomes were analyzed by SDS-PAGE followed by autoradiography. Figure 4 demonstrates that both fractions contain an appreciable proportion of the newly formed material that migrates as a single band approximately corresponding to native rhodanese (Mr = 33,000). The position of the band from the supernatant fraction was indistinguishable from that of rhodanese isolated from bovine liver. However, the autoradiogram showed that the corresponding band from the ribosome fraction migrates somewhat slower, corresponding to a 35,000 molecular weight polypeptide.

A number of newly formed shorter peptides were visible in the lane of the ribosome fraction. By excising then counting that portion of the gel containing the slowly moving band, it was determined that it contains about 60% of the incorporated [^{14}C]leucine that was present in the ribosome fraction. The remaining 40% was distributed between a number of smaller peptides, some of which can be seen as faint, somewhat diffuse bands. The supernatant fraction contains almost exclusively full-length rhodanese compared with rhodanese isolate from bovine liver.

The reason for the difference in the rate of migration of the full-length rhodanese in the supernatant vs. the ribosome fraction is not known. Removal of an N-terminal signal sequence does not occur *in vivo* for processing, transport and folding of native rhodanese. That the N-terminal methionine was not hydrolyzed *in vitro* was verified by the fact that labeled polypeptide was formed from [^{35}S]fMet-tRNA as the only source of labeled amino acid. The results (included for comparison in Figure 4) were similar to those obtained with [^{14}C]leucine-labeled rhodanese. The faster migrating form of active rhodanese in the supernatant

retains [^{35}S]methionine at its N-terminus. It has the same electrophoretic mobility and was indistinguishable on this basis from native rhodanese isolated from bovine liver. A possible explanation for the size difference shown in Figure 4 was that UGA, a weak terminator in *E. coli*, was read through. The coding sequence of rhodanese mRNA contains a UGA termination codon which is followed by 13 codons then an in-phase UAG termination codon. This explanation assumes proteolytic removal of sixteen amino acids as the polypeptide is released from the *E. coli* ribosome.

EXAMPLE 13

Activation of ribosome-bound rhodanese

Ribosomes isolated by centrifugation from a reaction mixture in which rhodanese had been synthesized in the absence of added chaperones (TABLE VII A) were incubated in a second reaction mixture which lacked components required for protein synthesis with the objective of characterizing the activation process. After incubation, these reaction mixtures were again subjected to centrifugation to separate ribosomes from the soluble fraction. All enzymatic activity was found in the supernatant fraction as observed after the first incubation. The clear but surprising results shown in TABLE VIII demonstrate that the chaperones promote release of ribosome-bound polypeptides and that active enzyme can be generated from these inactive rhodanese polypeptides. Both release and activation were very low in the absence of ATP under all of the conditions. The specific enzymatic activity of the material released in the presence of all of the chaperones indicates that nearly all of the rhodanese molecules were enzymatically active. Free amino acids, tRNA and the enzyme fraction containing the activating enzyme and peptide elongation

factors were not added to the reaction mixture. Although traces of the components may be present in the ribosome fraction, peptide elongation could not be detected during the second incubation.

TABLE VIII

Activation of ribosome-bound full-length rhodanese by chaperones

	In the supernatant after 2nd centrifugation		
Additions	[^{14}C]leucine in protein	enzymatic activity	specific
enzymatic			
	(pmol)	(units x 10^{-3})	activity
(units/mg)			
None	4	0.0	0
GroEl + GroES	21	0.07	2
DnaK + DnaJ	19	0.02	1
DnaK + DnaJ + GrpE	4	0.0	0
GroEL/ES + Dna K/J + GrpE	33	30.8	
679			
chaperones minus ATP, TGP	9	0.01	1
Puromycin	23	0.0	0
Puromycin; chaperones after 10 minutes	22	0.0	0
Sparsomychin; chaperones after 10 minutes	12	0.0	0

An aliquot of the ribosomal fraction (3 μl, 60 pmol [^{14}C]leucine in protein) isolated after coupled transcription/translation (see Methods) was incubated with

the indicated additions (amounts of the added chaperones as given above), plus low molecular weight components as specified above. Puromycin to give 1 mM and sparsomycin to give 4 μM were added where indicated. All reaction mixtures contained ATP and GTP unless indicated otherwise. Other compounds required for protein synthesis including amino acids were not added to this reaction mixture. After the 30 minute incubation, the ribosomes were separated from the supernatant by centrifugation. The amount of rhodanese released into the supernatant and its enzymatic activity were determined.

Individual chaperones were tested in different concentrations under the conditions given for Figure 5 or in combinations as in TABLE VIII. A high proportion of full-length rhodanese molecules were released from the ribosomes and converted to the faster migrating form during incubation with either GroES or DnaK (Figure 5) whereas DnaJ inhibited release. However, the material that was released with only GroES or DnaK was enzymatically inactive even though it was the faster migrating form. Thus, all chaperones were required together for activation and presumably to complete folding into the native conformation. In all of the situations shown in Figure 5, the molecules in the soluble fraction are of the faster moving type as discussed in relation to Figure 4. However, enzymatic activity was detected only in those samples which were incubated in the presence of all of the chaperones. Thus, generation of the faster moving polypeptide species was not correlated with the occurrence of enzymatic activity. No enzymatic activity with the slow moving species under any of the conditions was ever observed.

EXAMPLE 14

Inhibition of activation by puromycin and sparsomycin

The present invention raises several questions such as why the slow migrating, apparently full-length rhodanese polypeptides accumulate on the ribosomes and how they are bound to the ribosomes? Does their release require conventional termination? If translation is stalled at a pause site during peptide elongation that immediately precedes the release codon, they should exist as peptidyl-tRNA. If the peptides exist as peptidyl-tRNA in the ribosomal P site, then they should react with puromycin, an antibiotic that mimics A-site aminoacyl-tRNA resulting in a covalent bond to the nascent peptide during the peptidyl transferase reaction. If release and activation are dependent on the peptidyl transferase reaction, then these processes should be sensitive to inhibition by antibiotics such as sparsomycin that bind to the large ribosomal subunit and block this reaction. The effect of puromycin and sparsomycin on release and activation were tested with the results also shown in TABLE VIII. Ribosomes with bound polypeptides were incubated with either antibiotic with or without the delayed addition of the chaperones, then subjected to centrifugation as described above. About 40% of the rhodanese polypeptides that were isolated with the ribosomes after coupled transcription/translation were present in the soluble fraction after incubation with puromycin. The large polypeptides that were released were of the faster migrating species, but these had no enzymatic activity (Table VIII). No enzymatic activity was detected in the ribosomal fraction. Subsequent incubation of the polypeptides that had been released with puromycin, with

all of the chaperones plus ATP did not generate active enzyme.

A radioactively labeled analogue of puromycin was chemically synthesized (see above) and used to determine directly the reactivity of the ribosome-bound peptides. Incorporation of ^{3}H-labeled puromycin into peptides was determined and compared with the molar amount of peptide that was present, as calculated from incorporated radioactive leucine in a parallel sample by the procedure described above. 89% of the full-length polypeptides that were bound to the ribosomes after the initial incubation and centrifugation were reactive with puromycin and this material was present on the ribosomes as peptidyl-tRNA. Only 36% of these peptides were found in the soluble fraction after reaction with puromycin indicating that the puromycin-released polypeptides may aggregate, or for other reasons, remain associated with the ribosomes during centrifugation. In contrast to the results with puromycin, release was severely inhibited by sparsomycin. In the presence of this antibiotic, enzymatic activity was not detected in the supernatant fraction and the rhodanese polypeptides in the ribosome fraction remain enzymatically inactive even after incubation in the presence of all chaperones plus ATP (TABLE VIII).

EXAMPLE 15

Temperature sensitivity of chaperone effect

The temperature requirement for activation and release of ribosome-bound rhodanese by chaperones is shown in TABLE IX. About 4 pmol of rhodanese bound to ribosomes were incubated in a total volume of 30 ul under nontranslating conditions (but in the presence of ATP and GTP) in the absence or presence of chaperones for 10 minutes at the indicated temperature. Subsequently,

enzymatic activity was determined with a 20 ul aliquot from these incubations. Rhodanese was determined as described above. The amounts of chaperones added were as follows: DnaJ= 1 μg; DnaK= 2 μg; GrpE = 1.5 μg; GroEL= 3.2 μg; and GroES= 0.9 μg. As TABLE IX indicates, activation occurs only when the ribosomes were incubated with all of the chaperones in the presence of ATP and requires incubation at 25°C or 37°C.

TABLE IX

Temperature sensitivity of chaperone effect

Additions	Enzymatic activity after indicated temperature of incubation		
	0°C	25°C	37°C
None	0.0	0.0	0.0
Chaperones	0.5	5.1	8.0

The present invention demonstrates that at least part of the process by which newly formed rhodanese polypeptides are folded and converted into an enzymatically active state occurs on ribosomes. The process requires the five chaperones, DnaK, DnaJ, GrpE, GroEL and GroES, plus ATP and is temporally associated with release of the nascent protein from peptidyl-tRNA on the ribosome on which it was formed. Reaction with puromycin shows that the ribosome-bound newly formed peptides exist at the P site within the translational apparatus of the ribosome as peptidyl-tRNA. Inhibition of its release by sparsomycin supports this and indicates that the peptidyl transferase reaction is involved in its

release as is the case for codon-directed termination by the well-characterized release factors. That both the ribosome-bound material and its puromycin-released counterpart have no detectable enzymatic activity suggests that some portion of the folding and activation process has not been completed with the full-length polypeptides that are accumulated on the ribosomes. The present invention is supported by the fact that high specific enzymatic activity of the product was released in the presence of added chaperones without incorporation of additional amino acids. Thus, the present invention shows that accumulation of full-length polypeptides on the ribosome results from a failure in some part of the termination and release mechanism which is coupled to final folding and activation of the enzyme. Termination and release of the peptides was inhibited by the addition of purified DnaJ to the reaction system which contains ATP, whereas either GroES or DnaK promoted release. Considered together, there is an intimate association between a late stage of folding mediated by chaperones and termination-release of a nascent protein from the ribosome on which it was formed.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments,

molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

Claims

1. A high efficiency method for the cell free synthesis of proteins, comprising the steps of:

preparing a cell free extract;

separating out a ribosome fraction from said extract;

incubating said ribosome fraction in the presence of a transcription/translation medium; and

measuring the amount of protein synthesized.

2. The method of claim 1, wherein the cell free extract is prepared from cells selected from the group consisting of *Escherichia coli*, wheat germ and reticulocyte.

3. The method of claim 1, wherein said ribosome fraction is separated from said cell free extract by a method selected from the group consisting of centrifugation and gel filtration chromatography.

4. The method of claim 1, wherein said medium is a coupled transcription/translation medium.

5. The method of claim 4, wherein said transcription/translation medium contains plasmid DNA, RNA polymerase, nucleoside triphosphates, an energy regenerating system and amino acids in a buffered salt solution.

6. The method of claim 5, wherein said plasmid DNA is non-linearized.

7. The method of claim 1, wherein said incubating is for about 30 minutes at about 37° C.

8. The method of claim 1, wherein said medium is a translation medium.

9. The method of claim 8, wherein said translation medium contains mRNA, ATP, GTP, an energy regenerating system and amino acids in a buffered salt solution.

10. The method of claim 1, wherein said protein is measured by a method selected from the group consisting of trichloroacetic acid precipitation followed by quantitation of the amount of amino acid incorporated into the protein and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography.

11. The method of claim 1, wherein the native conformation of said protein is determined by measuring the biological activity of the protein.

12. The method of claim 1, wherein said cell free extract is prepared from cells harvested in early log phase.

13. The method of claim 1, wherein said incubation mixture is part of a continuous flow system.

14. A high efficiency method for the cell-free synthesis of proteins, comprising the steps of:

preparing a cell-free extract from *Escherischia coli*;

separating out a ribosome fraction from said extract by centrifugation;

incubating said ribosome fraction in the presence of a medium, said medium comprising 55 mM Tris-acetate (pH 7.8), 14 mM Mg(OAc)2, 36 mM NH4OAc, 72 mM KOAc, 2 mM Ca(OAc)2, 0.5 mM EDTA, 2% polyethylene glycol-6000, 2 mM DTT, 1.2 mM ATP, 0.8 mM GTP, 0.8 mM UTP, 0.8 mM CTP, 0.4 mM cAMP, 27 mM phosphoenol pyruvate, 0.35 μg pyruvate kinase, 1 μg folinic acid , 83 μg ^{14}C-leucine, 330 μM of each of the other 19 amino acids, 20 μg E. coli tRNA, 0.5 μg rifampicin, 0.3 mM glucose-6-phosphate, 1.2 A_{260} units of the *E. coli* ribosome fraction 0.5 μg plasmid DNA and 0.5 μg SP6 RNA polymerase; and

measuring the amount of protein synthesized and its biological activity.

15. A high efficiency method for the cell free synthesis of proteins, comprising the steps of:

preparing a cell free extract;

separating out a ribosome fraction from said extract;

incubating said ribosome fraction in the presence of a transcription/translation medium containing chaperone proteins; and

measuring the amount of protein synthesized.

16. The method of claim 15, wherein said chaperones are selected from the group consisting of DnaJ, DnaK, GrpE, GroEL and GroES.

17. The method of claim 9, wherein said translation medium further contains chaperone proteins.

18. The method of claim 17, wherein said chaperones are selected from the group consisting of DnaJ, DnaK, GrpE, GroEL and GroES.

AMENDED CLAIMS

[received by the International Bureau on 12 September 1994 (12.09.94); original claim 1 amended; remaining claims unchanged (1 page)]

1. A high efficiency method for the cell free synthesis of proteins, comprising the steps of:

preparing a cell free extract;

separating out a ribosome fraction from said extract;

incubating said ribosome fraction in the presence of a transcription/translation medium, said ribosome fraction essentially free of soluble enzymes that degrade proteins and nucleic acids; and

measuring the amount of protein synthesized.

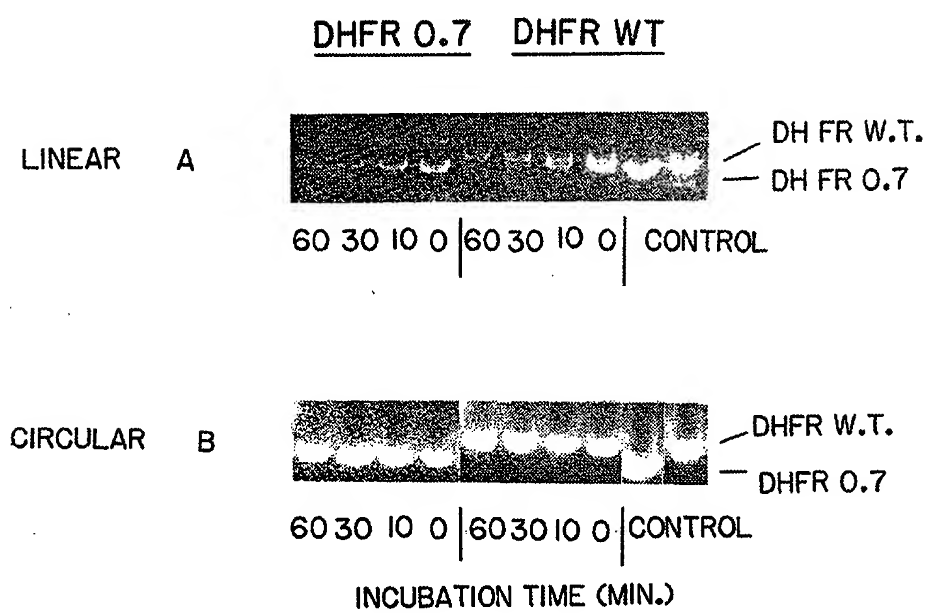
2. The method of claim 1, wherein the cell free extract is prepared from cells selected from the group consisting of *Escherichia coli*, wheat germ and reticulocyte.

3. The method of claim 1, wherein said ribosome fraction is separated from said cell free extract by a method selected from the group consisting of centrifugation and gel filtration chromatography.

4. The method of claim 1, wherein said medium is a coupled transcription/translation medium.

5. The method of claim 4, wherein said transcription/translation medium contains plasmid DNA, RNA polymerase, nucleoside triphosphates, an energy regenerating system and amino acids in a buffered salt solution.

6. The method of claim 5, wherein said plasmid DNA is non-linearized.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DHFR WT
LINEAR A
DH FR W.T.
DH FR 0.7
60 30 10 0
60 30 10 0
CONTROL
CIRCULAR B
DHFR W.T.
DHFR 0.7
60 30 10 0
60 30 10 0
CONTROL
INCUBATION TIME (MIN.)

FIG. 1

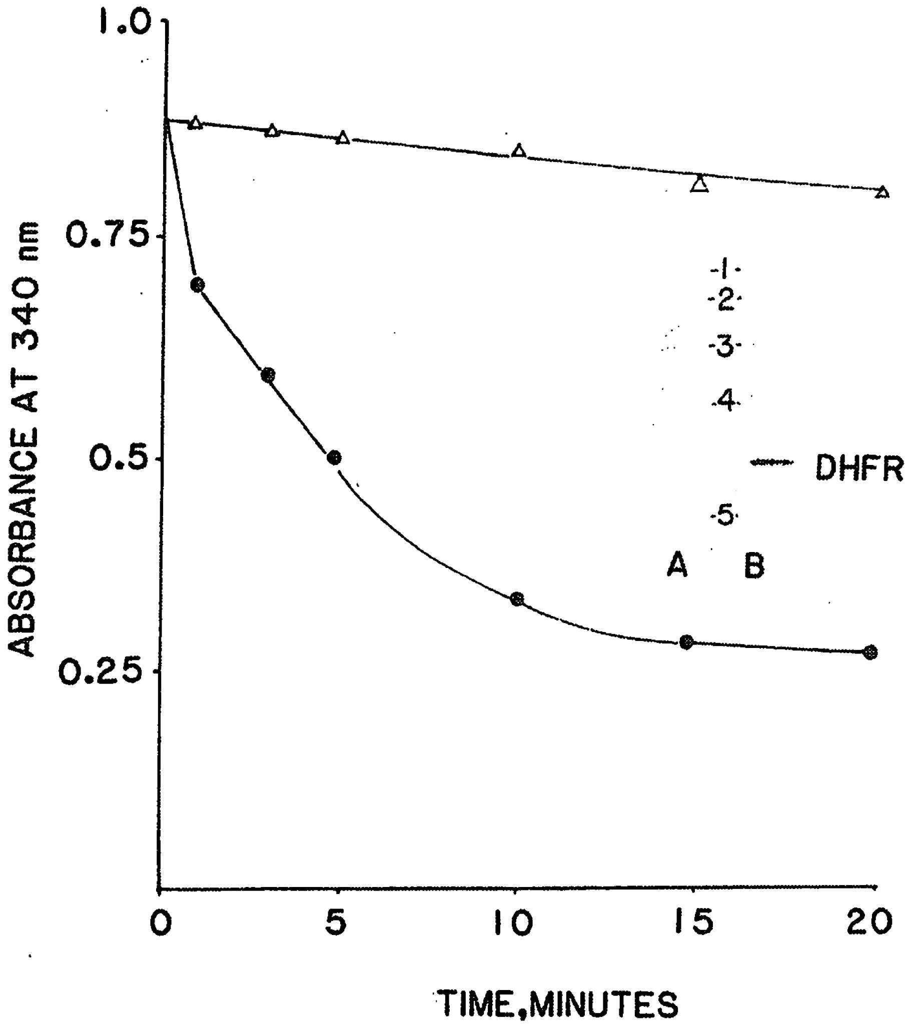

1.0
0.75
0.5
0.25
ABSORBANCE AT 340 nm
-1-
-2-
-3-
-4-
-5-
DHFR
A
B
0
5
10
15
20
TIME,MINUTES

FIG. 2

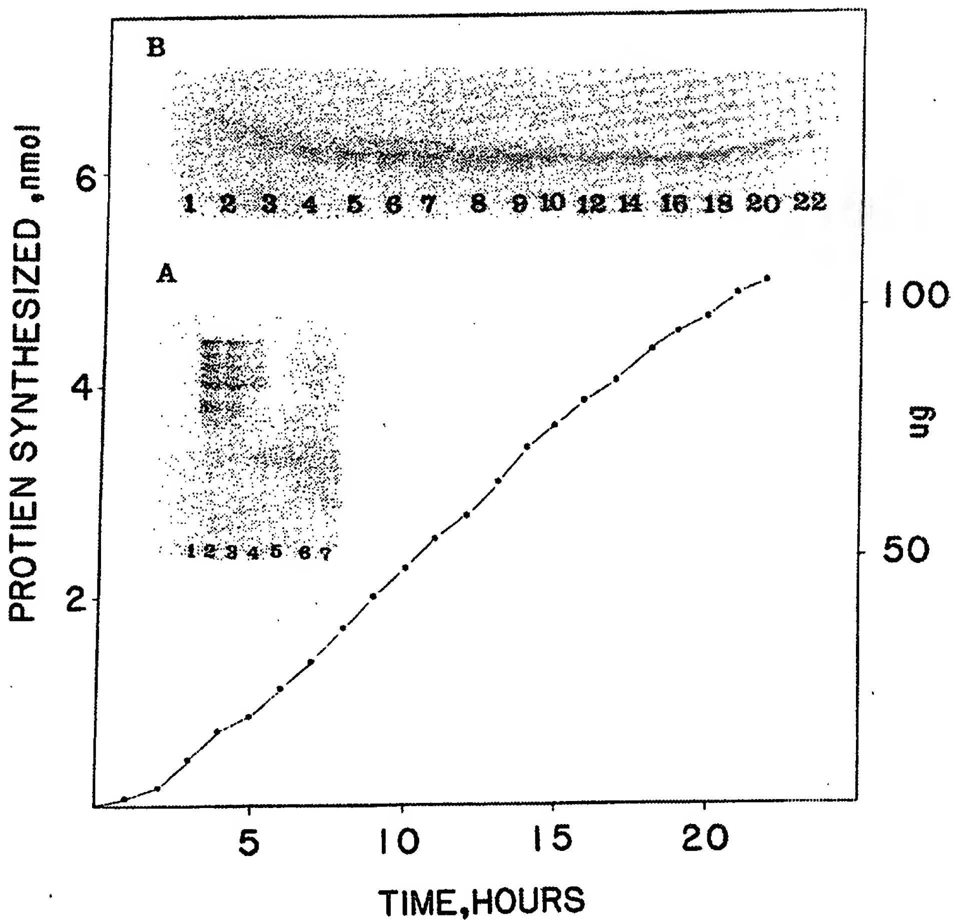

B
1 2 3 4 5 6 7 8 9 10 12 14 16 18 20 22
A
1 2 3 4 5 6 7
PROTIEN SYNTHESIZED ,nmol
6
4
2
100
50
ug
5
10
15
20
TIME,HOURS

FIG. 3

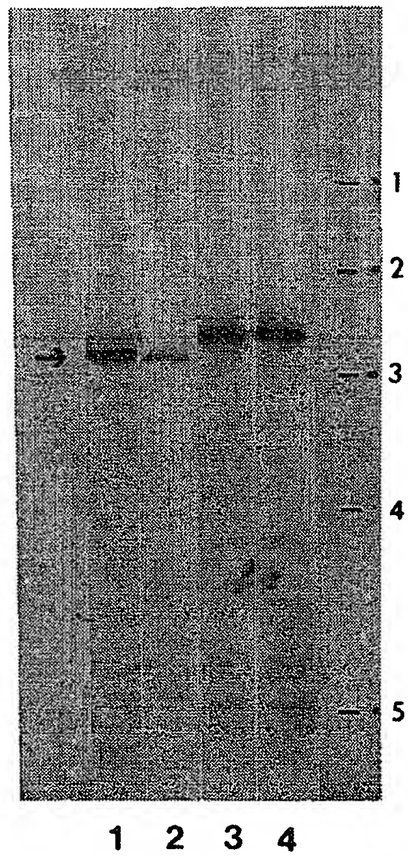
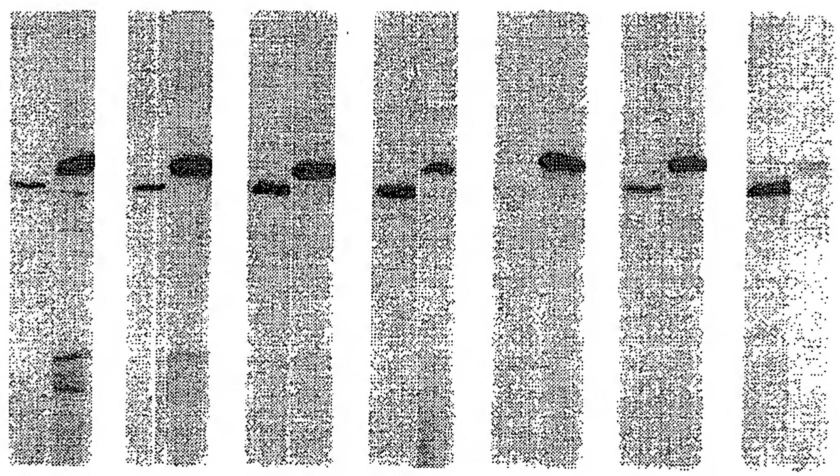


FIG. 4



ADDITIONS	NONE	GroEL	GroES	DnaK	DnaJ	GrpE	ALL
% RELEASED	7	9	50	55	2	10	90

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03860

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12P 21/00
US CL : 435/68.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/68.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Methods in Enzymology, Volume 101, issued 1983, Henshaw et al., "Translational Systems Prepared from the Ehrlich Ascites Tumor Cell", pages 616-629, see entire document.	1-18
Y	Methods in Enzymology, Volume 101, issued 1983, Chamberlin et al., "Isolation of Bacterial and Bacteriophage RNA Polymerases and Their Use in Synthesis of RNA in Vitro", pages 540-568, see entire document.	1-18
Y	Science, Volume 24, issued 25 November 1988, Spirin et al., "A continous Cell-Free Translation System Capable of Produing Polypeptides in High Yield", pages 1162-1164, see entire document.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 28 JUNE 1994	Date of mailing of the international search report JUL 11 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Gary L. Brown D. Kezza fa Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03860

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEBS, Volume 291, number 2, issued October 1991, Nevin et al., "A coupled in vitro transcription-translation system for the exclusive synthesis of peptides expressed from the T7 promoter", pages 259-263, see entire document.	1-18
Y	Methods in Enzymology, Volume 101, issued 1983, Chen et al., Prokaryotic Coupled Transcription-translation", see pages 674-690, see entire document.	1-18